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Identification of the genes and their polypeptide products responsible for aerobactin synthesis by pColV plasmids

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Summary. Iron acquisition via aerobactin enhances the virulence of *Escherichia coli*. Genes that specify functions for aerobactin synthesis and iron(III)-aerobactin transport have been identified on several ColV plasmids. Previously, we cloned the locus for aerobactin synthesis from pColV-K311 and assigned to three loci termed *aerA*, *aerB*, and *aerC* the functions for hydroxylation of lysine, acetylation of the 6-amino group of 6-hydroxy-lysine and coupling of N-acetyl-N-hydroxy-lysine with citrate, respectively (Gross et al. 1984). In this paper we show that *aerA* and *aerB* determine polypeptides with molecular weights of 50,000 and 35,000, respectively. We identified a fourth gene designated *aerD* that codes for a polypeptide with a molecular weight of 60,000, and which is required for the linkage of one residue of N-acetyl-N-hydroxy-lysine to citrate. The *aerC* gene product completes aerobactin synthesis by coupling the second N-acetyl-N-hydroxy-lysine to the monoacylated derivative citrate. The order of the genes in the operon was found to be *aerD*−*aerB*−*aerC*−*aerA*.

Introduction

In isolates of invasive *Escherichia coli* of human and animal origin a remarkable iron acquisition system has been detected which allows the bacteria to overcome the extreme iron deficiency of normal serum (Williams 1979; Stuart et al. 1980). Production of an iron-chelating compound and transport of iron(III) has been shown to be conferred by certain ColV plasmids (Braun 1985; Crosa 1984). The iron-chelating compound is aerobactin, originally characterized in *Aerobacter aerogenes*, which consists of two residues of 6-(N-acetyl-N-hydroxy)lysine linked to citrate (Gibson and Magrath 1969). We have studied the iron(III)-aerobactin system of pColV-K311 which was isolated from human feces by P. Frédericq, Liège. It specifies an outer membrane receptor protein necessary for aerobactin-mediated iron transport (Bindereif et al. 1982) and several genes for aerobactin synthesis (Braun 1981; Gross et al. 1984). In addition, the chromosomal *fhuC, D, B*, the *tonB* and the *exbB* genes are required for transport (Braun et al. 1982; Braun et al. 1983; Fecker and Braun 1983). By inserting Mud1 (Ap *lac*) into the synthesis and transport genes of pColV-K311, derepression of transcription was measured quantitatively and found to be controlled by the iron supply and the chromosomal *fur* gene (Braun and Burckhardt 1982). The latter gene regulates about 30 genes which exhibit iron-dependent expression (Hantke 1981; Schäffer et al. 1985). Furthermore, from the orientation of Mud1(Ap *lac*) insertions and the reduction of transcription by polar Tn*5* and Tn*1000* transposon insertions, the transcriptional polarity of the entire aerobactin region of pColV-K311 was deduced (Gross et al. 1984).

In addition to the outer membrane receptor protein, Krone et al. (1983) provided evidence that an additional protein with a molecular weight of 50,000 was required for iron(III)-aerobactin transport. The structural gene was mapped to the left of the *iut* gene for the outer membrane protein. This finding contradicted our model which predicts that all hydroxamate-type iron transport systems (aerobactin, ferrichrome, coprogen, rhodotorulic acid) require specific receptor proteins in the outer membrane but common functions for subsequent steps specified by the *fhuC, D, B, tonB* and *exbB* genes (Braun et al. 1983; Hantke 1983). We therefore reinvestigated the function of the 50,000 protein on the same ColV-K30 plasmid used by Krone et al. (1983). We show here that the 50,000 protein encoded by either the K30 or K311 isolates of pColV is not involved in transport but in fact is the hydroxylase encoded by the *aerA* gene. In addition, we detected on gels a polypeptide with a molecular weight of 60,000 which was encoded by a gene mapping to the left of the aerobactin biosynthesis genes hitherto identified (Gross et al. 1984). The data obtained suggest that this protein catalyses the penultimate step in aerobactin biosynthesis.

Materials and methods

Bacterial strains and plasmids. The *E. coli* strains used are listed in Table 1. The aerobactin biosynthesis region of pColV-K311 comprising the genes *aerA* to *D*, Tn*1000* transposon insertions or deletions thereof were cloned into pBR322 (Bolivar 1978), pACYC184 (Chang and Cohen 1978) or pUC9 (Vieira and Messing 1982). These plasmids are listed in Figs. 1 and 2. For cloning the *aerA-iut* region into pBR322, pColV-K30 from *E. coli* KH576 was employed. Strains H1443 and EN75 are *aroB* mutants of *E. coli*. They are unable to produce enterochelin (also called

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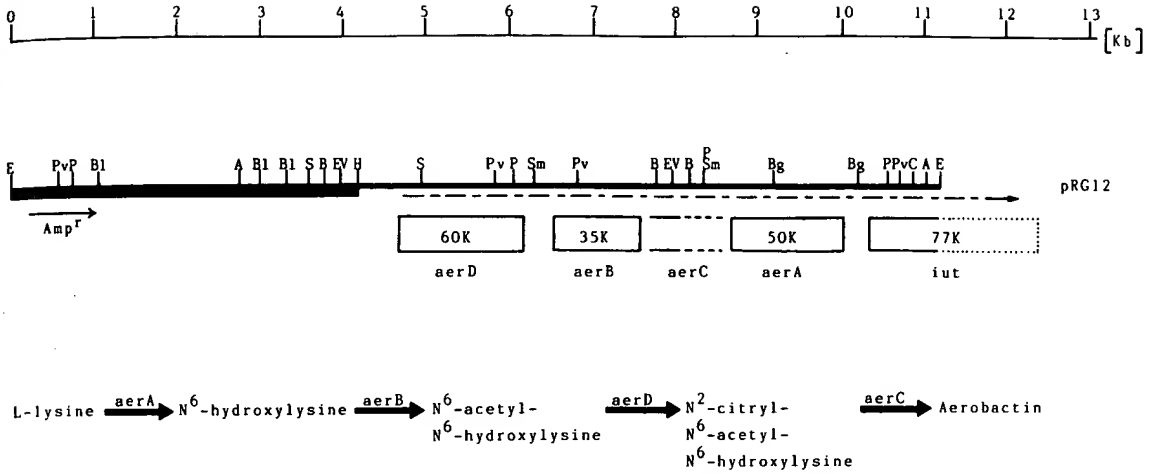


Fig. 1. Physical map, arrangement and functions of the *aer* genes of pRG12. The 60 K, 35 K and 50 K proteins are encoded by the *aerD*, *aerB* and *aerA* genes, respectively. The size of the 77 K protein was deduced from the nucleotide sequence of the *iut* gene (Krone et al. 1985) and is somewhat larger than the apparent 74.5 K molecular weight observed on polyacrylamide gels (Bindereif et al. 1982). The sequence of reactions catalysed by the various *aer* gene products, as suggested by this paper, is also drawn. Amp^r designates the ampicillin resistance determined by the vector pBR322 (*strong line*). Cleavage sites of restriction enzymes are marked A, *Ava*I; B; *Bam*HI; B1, *Bgl*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; *Ev*, EcoRV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*I; S, *Sal*I; Sm, *Sma*I. The *dashed arrow* indicates the transcriptional polarity

Table 1. Strains of *Escherichia coli* used

Strain	Genotype and properties	Source
K311	Wild type, containing plasmid ColV-K311	P. Frédericq
H1443	K12 strain, *aroB rpsL lac araD*	Gross et al. (1984)
EN775	H1443, pColV-K311::Tn*10*	Gross et al. (1984)
EN25, 27 29, 30, 45, 63	*aerC* derivatives of EN775	This study
EN38, 42	*aerD* derivatives of EN775	This study
KH576	K12 strain, pColV-K30	K. Hardy
EN302	H1443, pEN2 *aerA*$^+$ *iut*$^+$	K. Hardy
EN304	H1443, pEN4 *aerA*$^+$ *iut*$^-$	K. Hardy
EN307	H1443, pEN7 *aerA*$^-$ *iut*$^+$	K. Hardy
EN75	H1443, but *rpsE*	Gross et al. (1984)
DS410	*lacY rpsL xyl thi mtl minA minB*	

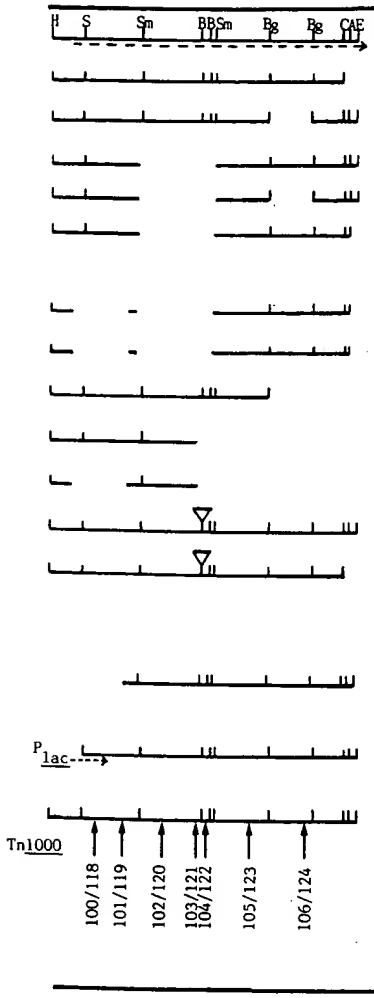
enterobactin) and thus are entirely dependent on the iron(III)-aerobactin system for growth in an iron-limiting growth medium. Expression of *aer* genes was studied in these strains.

Growth conditions. Cells were grown in tryptone-yeast extract medium which is rich in available iron so that addition of aerobactin was not required for growth of *aer* mutants. Iron limitation was imposed by adding 0.2 mM 2,2′dipyridyl as a nonutilizable iron chelating compound to iron-poor nutrient broth. Mutants (10^8 cells) of aerobactin synthesis were seeded on nutrient broth-dipyridyl agar plates and growth promotion was tested around filter paper disks (6 mm diameter) either impregnated with 15 µl 1 mM aerobactin, or by placing 10 µl of an overnight culture of the aerobactin-producing strain EN775 onto the filter (Braun et al. 1983). Complementation of mutants was tested by the same procedure. Maintenance of the plasmids was achieved by adding the antibiotics to which they conferred resistance.

Recombinant DNA techniques. Isolation of plasmids, cutting with restriction enzymes, ligation, agar gel electrophoresis and transformation followed basically the procedures published by Maniatis et al. (1982) and have been described in more detail in earlier publications (Fecker and Braun 1983; Braun et al. 1983; Gross et al. 1984). The deletions in pRG140, 137 and 142 were obtained by cleaving pRG133 with *Sal*I, pRG132 with *Pst*I, and pRG118 with *Xho*I, respectively, and subsequent treatment with Bal31 (Fig. 2). The deletion in pRG134 was created by excising the fragment between the two *Bgl*II sites and that of pRG133 by cloning the *Ava*I/*Hin*dIII fragment of pRG111 into pACYC184. The deletion of pRG145 was formed by cleaving pRG12 with *Bgl*II and *Hin*dIII and ligation into pACYC184 cleaved with *Bam*HI/*Hin*dIII and that of pRG136 by cleaving pRG111 with *Bgl*II. Plasmid pRG141 was derived from pRG140 by cutting with *Ava*I/*Hin*dIII and cloning into pBR322 (Fig. 2).

Determination of plasmid-encoded polypeptides. Minicells were prepared from the minicell-producing *E. coli* strain DS410 which had been transformed with various plasmids. Labelling with a mixture of [^{14}C]amino acids or [^{35}S]-methionine and subsequent electrophoresis on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate was done as described previously (Fecker and Braun 1983).

Identification of aerobactin precursors. The precursors released from cells into the culture medium were analysed. The N-hydroxy group of N-hydroxy-lysine was determined colorimetrically after oxidation with iodine (Tomlinson et al. 1971). The N-acetyl-N-hydroxy group of N-acetyl-N-



Plasmid pRG	Genotype aer	Synthesis of	Synthesis of aerobactin or of intermediate compounds after complementation with pRG plasmids			
			HL	AL	CL	Aer
12[a]	$A^+B^+C^+D^+$	Aer				
13[b]	$A^+B^+C^+D^+$	Aer				
134[a]	$A^-B^+C^+D^+$	-				131 133
111[a]	$A^+B^-C^-D^+$	HL			132	145
136[a]	$A^-B^-C^-D^+$	-				
133[b]	$A^+B^-C^-D^+$	HL	101 102		103 104	105 106 134 142
140[b]	$A^+B^-C^-D^-$	HL				
141[a]	$A^+B^-C^-D^-$	HL		137		
145[b]	$A^-B^+C^+D^+$	-				111 128
132[b]	$A^-B^+C^-D^+$	-			111 128	142
137[b]	$A^-B^+C^-D^-$	-		141 142		
128[a]	$A^+B^+C^-D^+$	CL			132	145
131[b]	$A^+B^+C^-D^+$	CL			103 104	101 102 105 106 134 142
142[a]	$A^+B^+C^+D^-$	AL		137		131 132 133
144[c]	$A^+B^+C^+D^-$	AL				
100[a]	$A^+B^+C^+D^-$	-				
101[a]	$A^+B^+C^+D^-$	-	133			131
102[a]	$A^+B^-C^+D^+$	-	133			131
103[a] 104[a]	$A^+B^+C^-D^+$	-			133 131	
105[a] 106[a]	$A^-B^+C^+D^+$	-				131 133

Fig. 2. List of the plasmids, their physical map and the functions they specify. The DNA of the vectors (pBR322, marked [a] and pACYC184, marked [b]) is not drawn. For comparison see Fig. 1. P_{lac} marks the *lac* promoter on pUC9. pRG128 and pRG131 contain a duplication of the short *Bam*HI fragment. The *arrows* mark the Tn*1000* insertions in plasmids 100 to 106 from which the plasmids 118 to 124 were derived by excising the *Xho*I fragment in Tn*1000*. The products released by cells containing the plasmids are listed and also the products formed when an additional plasmid was introduced into the cells. Aer, aerobactin; AL, N^6-acetyl-N^6-hydroxylysine; CL, N^2-citryl-N^6-acetyl-N^6-hydroxy-lysine; HL, N^6-hydroxylysine

hydroxy-lysine was determined as described by Csaky (1948) and Gibson and Magrath (1969); details have been given in a preceding paper (Gross et al. 1984). Cells were grown in an iron-poor minimal medium (M56) that consisted of 13.6 g NH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 6\,H_2O$, 0.01 g $CaCl_2$, 100 mg shikimic acid and 4 g glucose per litre, adjusted to pH 7.4 with KOH. When 20 µM $FeCl_3$ was added to the medium, no aerobactin or precursors were detectable in the culture supernatant. The concentration of precursors was increased by a factor of 2.8 by adding 1 mM lysine to the growth medium. N^6-hydroxy-lysine was also determined by chromatography on silica gel (number 60) thin layer plates (Merck, Darmstadt) with the system ethanol/H_2O = 70:30 and showed a ninhydrin-positive spot with an R_f value of 0.45, the same as the synthetic reference compound. The latter was synthesized by Jauch and Naegeli at Sandoz AG, Basel. Ten ml of a fivefold concentrated culture supernatant were chromatographed on a 100 ml column of Dowex 50WX8. The column was washed successively with 10 ml H_2O, 10 ml 1 N HCl, 10 ml 2 N HCl. The N-hydroxy-lysine was then eluted with 10 ml 3 N HCl. After rotary evaporation the dried material was dissolved in 2 ml H_2O and subjected to thin-layer chromatography and the colorimetric assay of Tomlinson et al. (1971). N-acetyl-N-hydroxy-lysine was also determined quantitatively on the amino acid analyser (Gross et al. 1984).

For the determination of N^2-citryl-N^6-acetyl-N^6-hydroxy-lysine, 200 ml of the supernatant of an overnight culture of strain GR111 was chromatographed on a column of BioRad AG 1X2. The column was washed with 50 ml

H_2O, 50 ml 40 mM $MgCl_2$, and then with 100 ml 0.2 M $MgCl_2$. A hydroxamate-positive compound (Csaky test) was not retained on the column and was identified on the amino acid analyser as N-acetyl-N-hydroxy-lysine. The fraction eluted with 0.2 M $MgCl_2$ was also hydroxamate positive. It was concentrated by rotary evaporation and dissolved in 20 ml H_2O. Five ml were added to 5 ml 6 N HCl and heated for 60 min at 130° C. The hydrolysate was evaporated and this was repeated twice after addition of 5 ml H_2O. The residue was dissolved in H_2O, neutralized with NaOH and then adjusted to 5 ml by addition of H_2O. Citrate was determined in a sample of 0.1 ml using the enzymatic test kit for citrate of Boehringer, Mannheim (number 139076). The N-acetyl-N-hydroxy-lysine residue was determined by the Csaky assay with the modifications described previously (Gross et al. 1984).

Transport assays. Strains EN302, 304, 305 and 307 were grown overnight either in M56 medium or in tryptone-yeast extract medium. They were diluted to a density of 2×10^8 cells per ml and cultivated until 5×10^8 cells per ml was reached. Cells were harvested by centrifugation, spun down, washed once with M56 salts that contained 0.1 mM nitrilotriacetate and suspended again in this medium (5×10^8 cells per ml). (Nitrilotriacetate binds iron which cannot be used by *E. coli.*) Transport was started by addition of 0.1 µM ^{55}Fe (specific activity 0.4 GBq per µmol) that had been incubated overnight with 0.75 µM iron-free aerobactin. Samples of 0.1 ml were taken at the time intervals given in Fig. 5, filtered, washed twice with 5 ml 0.1 M LiCl, then the filters were dried and counted.

Results

Identification of proteins encoded by the aerobactin region

To lead the reader through the data we present in Fig. 1 the model derived from the results described in this paper. Plasmid pRG12 contains a *Hin*dIII/*Eco*RI fragment of pColV-K311 cloned into pBR322. It encodes all the enzymes required for aerobactin biosynthesis but does not determine iron(III)-aerobactin transport since *Eco*RI cuts in the *iut* gene that codes for the outer membrane receptor protein (Gross et al. 1984). The plasmids used for the expression of proteins in minicells are listed in Fig. 2. In Fig. 3 proteins expressed by *aer* gene insertions in pBR322 are listed on the left, those in pACYC184 are listed on the right. Minicells containing pRG13, which encompasses the entire *aer* region, contained proteins with molecular weights of 60,000 (60 K), 50,000 (50 K), 35,000 (35 K) and 27,000 (27 K) in addition to the chloramphenicol acetyltransferase (22 K) of the vector pACYC184. Localization of the coding regions for the 50 K and 60 K proteins were derived from pRG133 (Figs. 2 and 3) which determines both proteins, whereas only the 50 K protein was encoded by pRG140 and both were absent from pRG137. The Tn*1000* insertions in the *aer* genes cloned into pBR322 support these localizations.

The Tn*1000* insertion mutants pRG118 to pRG124 were derived from the previously described series pRG100 to 106 by deleting the *Xho*I fragment of Tn*1000*. In addition to the proteins expressed by minicells that contained the vector pBR322 (left hand lane, Fig. 3), pRG118- and 119-containing cells gave rise to two weakly expressed proteins with apparent molecular weights of 45,000 (45 K) and 50,000 (50 K) (Fig. 3). Plasmids pRG120 and 121 with Tn*1000* insertions further downstream (Fig. 1), determined two proteins of 50 K (weak) and 60 K (Fig. 3), whereas cells with pRG123 and 124 contained the 60 K but were lacking the 50 K protein. The fact that pRG133 (Fig. 3) and pRG111 (not shown) with a deletion between the two *Sma*I sites (Fig. 2) expressed the 60 K protein corroborates the assignment of the coding region for the 60 K protein to the left of the *Sma*I site, as deduced from the analysis of the Tn*1000* insertions in pRG118, 119 and 120 (see Fig. 1). The Tn*1000* insertions of pRG123 and 124 define the coding region for the 50 K protein shown in Fig. 1; this is supported by the lack of the 50 K protein in pRG145 with a deletion between the two *Bgl*II sites (Figs. 2 and 3).

Minicells containing pRG13, 137 (Fig. 3) and 131 (not shown) expressed a 35 K protein which was absent in cells

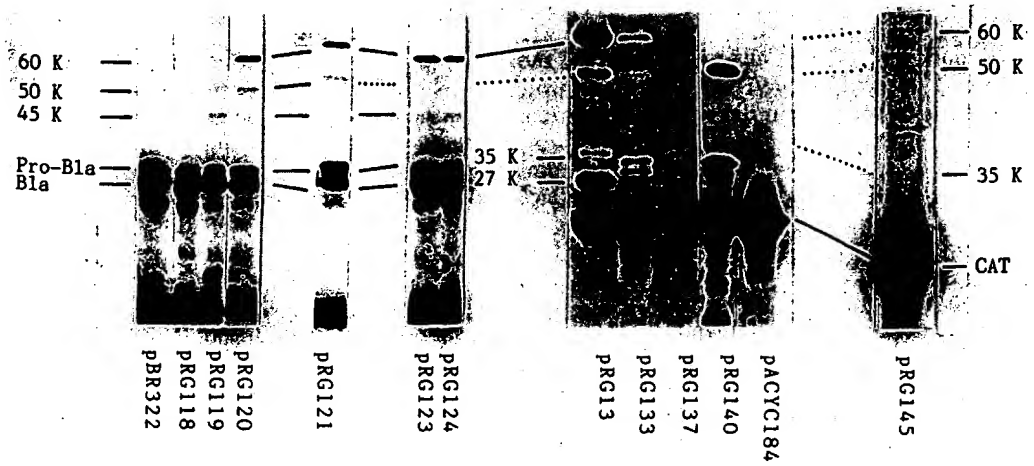


Fig. 3. Radioactively labelled proteins synthesized in minicells containing the plasmids listed at the bottom of the Figure (see also Fig. 2). Pro-Bla indicates the proform of the periplasmic β-lactamase (Bla) encoded on the vector pBR322. CAT designates the chloramphenicol acetyltransferase encoded by the vector pACYC184

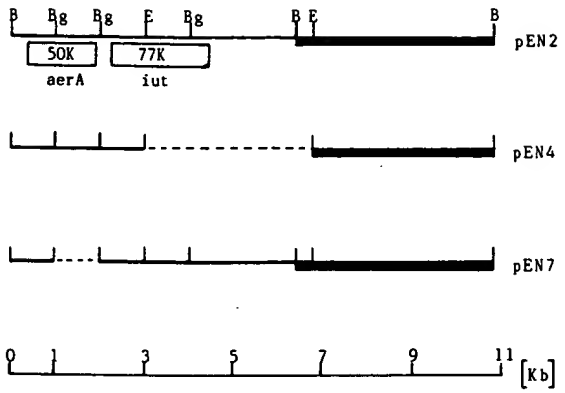


Fig. 4. Restriction fragments excised from pColV-K30 and cloned into pBR322. The *dashed lines* indicate deletions, the *thick line* the vector DNA. See Fig. 1 for abbreviations

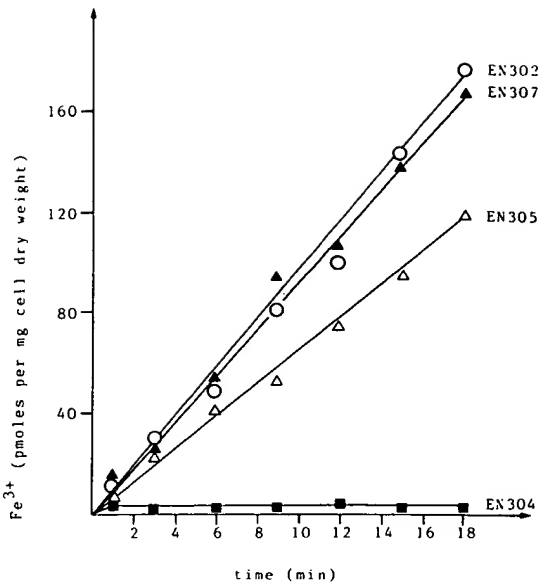


Fig. 5. Transport of iron(III) supplied as aerobactin complex into cells of EN302, 304, 305 and 307 that contained the plasmids pEN2 *aerA*$^+$*iut*$^+$, pEN4 *aerA*$^+$ *iut*$^-$, pEN7 *aerA*$^-$*iut*$^+$, respectively, of Fig. 4. EN305 contained pEN5 which consists of a *Hin*dIII fragment of pColV-K30 cloned into the *Hin*dIII site of pBR322. It comprises the entire region of pRG12 and extends to the right to the next *Hin*dIII site on pColV-K30. Logarithmically growing cells in tryptone-yeast extract medium were harvested, washed in M56 medium that contained 0.1 mM nitrilotriacetate and then resuspended in this medium before transport was started by addition of $^{55}Fe^{3+}$-aerobactin

bearing pRG133 (Fig. 3). Minicells carrying pRG132 synthesized the 60 K and 35 K proteins (data not shown), minicells with pRG145 formed the 60 K and 35 K proteins but no 50 K protein (Fig. 3). From these results we conclude that the 35 K protein is a genuine product of the aerobactin synthesis region. Since the 35 K protein was specified together with the 60 K protein by pRG132 but not by pRG133, the gene for the 35 K protein should follow that of the 60 K protein as depicted in Fig. 1.

In minicells with the pACYC184 derivatives, additional protein bands could be observed (Fig. 3). However, we assume that the 27, 31 and 32 K proteins are not genuine products and that they may be derived from *iut* gene fragments, since pRG13 and 131 cut at the *Cla*I site yielded the shortest polypeptide (27 K) and pRG133 and 140 cut at the *Ava*I site yielded the larger ones (31 and 32 K). A 45 K protein was weakly expressed by most plasmids and could not be assigned to a defined region (see Discussion).

Assignment of biosynthetic functions to the 50 K and 60 K proteins

The 50 K protein. Previously we (Gross et al. 1984) located the first gene for the lysine N-hydroxylase, *aerA*, close to the coding region for the 50 K protein for which a transport function had been claimed (Krone et al. 1983). Cells with Tn*1000* insertions in pRG123 and 124 and the *Bgl*II deletion in pRG134 which expressed no 50 K protein did not release N-hydroxy-lysine into the growth medium nor could aerobactin or a precursor be detected. Since these transposon insertions and deletions were at the position where Krone et al. had mapped the 50 K protein on pColV-K30 we cloned this gene region from this plasmid carried by strain KH576 to investigate whether the 50 K protein was required for transport and/or for the hydroxylation of lysine. Plasmid pColV-K30 was cleaved with *Bam*HI and cloned into pBR322 (Fig. 4). The fragment that encodes the 77 K outer membrane protein which serves as receptor for cloacin DF13 and aerobactin (Bindereif et al. 1982; van Tiel-Menkveld et al. 1981), also encodes the 50 K protein (Krone et al. 1983). Strain H1443 of *E. coli* was transformed and cloacin-sensitive transformants were obtained. The resulting strain EN302 used iron(III)-aerobactin as sole iron source and transported iron efficiently (Fig. 5); it did not produce aerobactin. Restriction analysis of pEN2 of strain EN302 showed identical sites (Fig. 4) to those published by Krone et al. (1985). Cutting out the *Eco*RI fragment of pEN2 resulted in pEN4 (Fig. 4), unable to confer growth on iron(III)-aerobactin as sole iron source or aerobactin-mediated iron transport (Fig. 5). When the 1 kb *Bgl*II fragment was excised from pEN2, the resulting pEN7 (Fig. 4) was able to confer the use and transport of iron as aerobactin complex (Fig. 5). The restriction fragments of the plasmids pEN2, pEN4, and pEN7, cleaved with *Bgl*II are shown in Fig. 6, left-hand panel.

To show that pEN2 encoded both the 50 K and the 77 K protein, pEN4 only the 50 K protein and pEN7 only the 77 K protein, the minicell-producing strain DS410 was transformed with the plasmids. The proteins were expressed in the minicells as expected (Fig. 6, right-hand panel). These results clearly demonstrate that the 77 K protein is the only plasmid-coded function necessary for the transport of iron(III)-aerobactin into cells and that the presence or absence of the 50 K protein does not affect transport.

The 60 K protein. To demonstrate that the 60 K protein is required for aerobactin synthesis we used various approaches. Strains containing plasmids with the Tn*1000* insertions 100 and 101 (Fig. 2) did not release detectable amounts of aerobactin or precursors (Gross et al. 1984). This could have arisen from polar effects since the 50 K protein was also very weakly expressed in these mutants (Fig. 3). In fact, all of the Tn*1000* insertions along the previously defined aerobactin synthesis region prevented for-

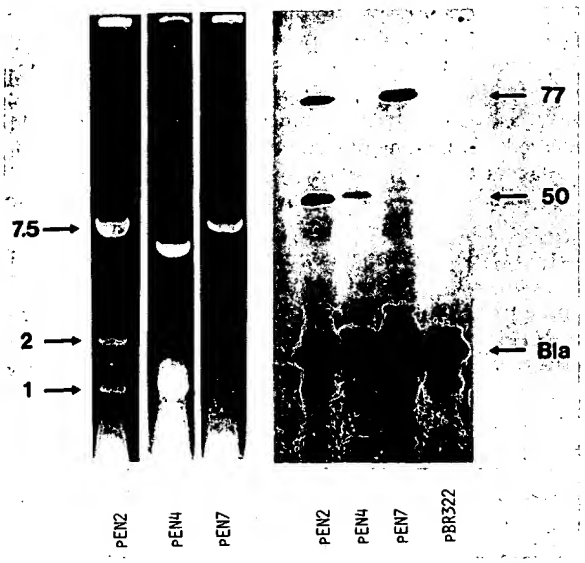


Fig. 6. *Left-hand panel: Bgl*II restriction fragments (kb) of pEN2, pEN4 and pEN7, respectively. Note the three fragments of pEN2, the two fragments, one smaller than in pEN2, of pEN4 and the two fragments of pEN7, both identical in size to the same fragments of pEN2, and the smallest one lacking in pEN7. *Right-hand panel:* proteins (k) expressed in minicells that contained the plasmids pEN2, pEN4 and pEN7, respectively. BLa, β lactamase

mation of any detectable aerobactin precursors, showing the strong polar effects of transposon insertions (the first biosynthetic gene is at the right-hand end). For this reason the *Sal*I/*Eco*RI fragment of pRG12 was cloned into pUC9. Cells carrying the resulting plasmid pRG144 (Fig. 2) did produce N-acetyl-N-hydroxy-lysine. Since this plasmid was lacking part of the gene for the 60 K protein, this protein must be required for a subsequent biosynthetic step. Henceforth, the structural gene for the 60 K protein will be designated *aerD*. The same result was obtained with pRG142 which was obtained by cleaving pRG118 at the single *Xho*I site remaining in Tn*1000* and subsequent treatment with *Bal*31. The resulting deletion extended to the left into the pBR322 vector and ended at the right before the *Sma*I site (Fig. 2). Furthermore, when a pRG142-containing strain was transformed with pRG131, pRG132 or pRG133, aerobactin was synthesized (Fig. 2). In contrast, pRG137 with a deletion in *aerD* was unable to complement pRG142 for aerobactin synthesis (Fig. 2).

Previously we have shown that the *aerC* gene (Fig. 1) is required for converting N-acetyl-N-hydroxy-lysine to aerobactin (Gross et al. 1984). We have now shown that a second gene, *aerD*, and its product, the 60 K protein, is also required for this reaction. Either two polypeptides form a heteromeric enzyme or one polypeptide is involved in the activation of the lysine derivative and the other one for citrate. Alternatively, the ligase activity may be separate from the activation enzyme or the reaction may proceed in two steps: linkage of one residue of N-acetyl-N-hydroxy-lysine to citrate may be catalysed by one polypeptide and transfer of the second lysine derivative to N^2-citryl-N^6-acetyl-N^6-hydroxy-lysine by the other polypeptide. To show that, indeed, two genes are required and to get an idea about the reaction sequence, we isolated additional mutants of pColV-K311, performed complementation analyses with

Table 2. Determination of hydroxamate-positive compounds after separation of culture supernatants of *aerC* and *aerD* mutants on a column of BioRad AG 1X2

Fractions of the eluate	*aerC* mutants			*aerD* mutants	
	EN25	EN27	EN63	EN38	EN42
35 ml culture supernatant + H_2O	0.8	1.3	1.2	1.7	2.1
30 ml H_2O	0.11	0.10	0.11	0.13	0.12
20 ml 40 mM $MgCl_2$	0.10	0.11	0.12	0.10	0.12
20 ml 40 mM $MgCl_2$	0.09	ND	ND	ND	ND
20 ml 0.2 M $MgCl_2$	0.30	0.58	0.50	0.09	0.10

The values represent the absorbance at 520 nm (Csaky test); ND, not determined

plasmids that contained various parts of the aerobactin region, and identified the products formed.

Eight mutants of pColV-K311, obtained by mutagenesis with N-methyl-N′-nitro-N-nitroso-guanidine, synthesized a hydroxamate derivative of lysine but no aerobactin. They were transformed into strains carrying pRG103, with a Tn*1000* insertion in *aerC*, or pRG142 with a deletion in *aerD*. Six of the mutant plasmids, among them the previously used *aerC*-type strain EN25 (Gross et al. 1984), were complemented for aerobactin synthesis by pRG142, but not by pRG103, and two were only complemented by pRG103. The *aerC* mutants were designated EN25, 27, 29, 30, 45, 63 and the *aerD* mutants EN38 and 42. In addition, EN25 was capable of weak aerobactin synthesis when complemented by pRG101 and 102, strong aerobactin synthesis with pRG106 and 142 and remained aerobactin-negative when transformed with pRG111, 113 or 128 [compare Fig. 1 with Fig. 2; pRG113 is a deletion extending from *aerB* to *aerA* (Gross et al. 1984)]. EN42 was transformed to aerobactin synthesis by pRG102, 111, 113 and 128 but not by pRG101. These results clearly prove the involvement of both *aerC* and *aerD* in the conversion of N-acetyl-N-hydroxy-lysine to aerobactin and they support the localization of the two genetic regions.

To examine whether the two classes of mutants secrete different precursors of aerobactin, the culture filtrates of the *aerC* mutants EN25, 27 and 63, and those of the *aerD* strains EN38 and 42, were chromatographed on an anion exchange column (BioRad AG 1X2). The water eluate of all the mutants was (in the first fractions) hydroxamate positive. On an amino acid analyser the hydroxamate had the same retention time as synthetic N^6-acetyl-N^6-lysine. The *aerC* mutants yielded a second hydroxamate-positive fraction which was eluted with 0.2 M $MgCl_2$. This negatively charged hydroxamate could not be detected on the amino acid analyser. N^2-citryl-N^6-acetyl-N^6-hydroxy-lysine would account for these properties. The quantitative values obtained with the various mutants are listed in Table 2.

The presence of citrate in the anionic hydroxamate compound was determined enzymatically after acid hydrolysis. The hydroxamate compound was isolated from the culture supernatant of a strain containing the multicopy plasmid pRG128 *aerC*. It produced twice the amount of N-acetyl-N-hydroxy-lysine and three to four times more of the new hydroxamate compound than the pColV-K3111 mutants

Table 3. Content of citrate in the hydrolysate of the anionic hydroxamate

Hydrolysate of strain	Citrate added	Absorbance
GR128	–[b]	0.34
GR128[a]	–	0.69
EN42	–	0.03
EN42	1 mM	0.22 (0.21)[c]
EN42	2 mM	0.43 (0.41)
EN42	3 mM	0.65 (0.61)

[a] Twice the amount used
[b] No addition
[c] The numbers in brackets were obtained with pure citrate in H_2O

listed in Table 2, probably due to the effect of gene dosage. Strain EN42 served as a negative control. After acid hydrolysis, citrate was determined in amounts (160 µM in the original culture supernatant) that were similar to the spectroscopically determined concentration of the hydroxamate (170 µM). In Table 3 the actual figures are given for EN42 and after addition of citrate to the EN42 supernatant. The figures in brackets represent the values obtained with a pure citrate solution. They show that the culture supernatant did not disturb the determination. The results demonstrate that *aerC* mutants accumulate the immediate precursor of aerobactin. We therefore conclude that the *aerC* gene product converts N^2-citryl-N^6-acetyl-N^6-hydroxy-lysine to aerobactin.

Discussion

Four genes could be assigned to steps in aerobactin synthesis determined by pColV-K311. Proteins with molecular weights of 50 K, 35 K and 60 K were shown to be encoded by the *aerA*, *aerB*, and *aerD* genes, respectively. The 45 K protein was weakly expressed by minicells whether or not they contained plasmids with mutants in the *aerC* or in any other *aer* gene region. Therefore, we do not know whether this protein is a genuine product encoded by an aerobactin synthesis gene.

Previously, we had localized the *aerA* gene to the immediate vicinity of the region that encodes the 50 K protein. The localization of the *aerA* gene was derived from the position of three transposon insertions within *aerA*. Since the size of the *aerA* gene product was not identified, the precise boundaries of the gene remained unknown. The transport function of the 50 K protein and the location of the gene were taken from Krone et al. (1983). The same laboratory has now sequenced the *iut* gene for the 77 K outer membrane receptor protein with the consequence that the 77 K and 50 K region has been moved somewhat to the left (Fig. 1; Krone et al. 1985), so that the 50 K region now overlaps with our *aerA* location. Since transposon insertions in *aerA* (pGR105, 106, 107) and deletions (pEN7, pRG145) did not release N-hydroxy-lysine, and were unable to complement the N-hydroxy-lysine-negative pColV-K311 mutant EN32, and since they were devoid of the 50 K protein (minicells containing pRG123, 124, 137, 145, Fig. 3; pEN7, Fig. 6), it is concluded that *aerA* encodes the 50 K protein and is essential for hydroxylation of lysine.

The location of *aerA* at the right-hand side of the *aer* region was also supported by the finding that none of the transposon insertions along the entire *aer* region released any chemically detectable precursor. Their polar effect reduced the formation of the first product of the biosynthetic pathway, N-hydroxy-lysine, such that no precursor could be observed. Complementation of mutants in *aerA* with $aerA^+$ plasmids led without exception to synthesis of aerobactin or its precursors, depending on the combination of genes introduced into the cells (Fig. 2). Moreover, Diekmann and Heydel (personal communication) have obtained partially purified lysine N-hydroxylases from strain GR111 containing pRG111 $aerA^+D^+$ and from strain GR141 carrying pRG141 $aerA^+$ with specific activities 14 and 3 times, respectively, higher than that found in *Aerobacter aerogenes* 62-1. Furthermore, the *aerA* deletion mutant EN7 (pEN7 iut^+) transported iron(III), delivered as aerobactin complex, with a rate similar to strains that were $aerA^+$ iut^+ (Fig. 5). Therefore, we concluded that the Iut outer membrane receptor protein is the only transport function encoded by pColV-K311.

In a former publication, we described one gene, *aerC*, that was required for conversion of N-acetyl-N-hydroxy-lysine to aerobactin (Gross et al. 1984). This conclusion is supported by the results of this paper because the mutant used in those studies, EN25, secretes both N-acetyl-N-hydroxy-lysine and its citryl derivative. Apparently, a portion of N-acetyl-N-hydroxy-lysine was secreted before it could be converted to the citryl derivative. Search for a function for the 60 K protein, encoded by the *aerD* gene, led to the identification of the stepwise conversion of N-acetyl-N-hydroxy-lysine to aerobactin. Firstly, mono-N-acetyl-N-hydroxy-lysyl-citrate is formed by the action of the *aerD* product and then *aerC* is necessary for linking the second N-acetyl-N-hydroxy-lysine group to the citrate derivative to yield aerobactin. This conclusion is based on the isolation of a new class of pColV-K311 mutants (EN38 and 42) and on the *aerD* deletion mutants GR142 and 144 which released only N-acetyl-N-hydroxy-lysine. GR142 was transformed to aerobactin synthesis by pRG131 $aerA^+$, B^+, D^+, pRG132 $aerB^+$, D^+ and pRG133 $aerA^+$, D^+ but not by pRG137 $aerB^+$, D^- (Fig. 2). The results obtained with additional plasmid combinations with deletions in *aerD* and other aer^+ genes (for example pRG137, 140, 141) were consistent with the localization and the function of the *aerD* gene (Fig. 2).

The clones with various combinations of the *aer* genes on multicopy plasmids, and the identification of the steps in aerobactin biosynthesis these gene products catalyse, will facilitate the elucidation of the enzymology of the whole process and the catalytic functions of the individual gene products. N-hydroxylation of lysine (Jackson et al. 1984), acetylation of N-hydroxy-lysine (Kusters and Diekmann 1984) and synthesis of aerobactin from citrate and N-acetyl-N-hydroxy-lysine (Appanna et al. 1984) have already been achieved with cell-free fractions from *Aerobacter aerogenes* 62-1 and *Klebsiella pneumoniae*.

Recently four polypeptides encoded by a cloned fragment of pColV-K30 were identified as determining aerobactin biosynthesis. The size and the order of the genes of the polypeptides was 62, 35, 45 and 50 K, yet the location of the gene for the 45 K protein remained uncertain. No functions were assigned to the individual genes in the pathway of aerobactin biosynthesis (Carbonetti and Williams 1984). These data agree with the results of this paper on

pColV-K311. These authors also conclude, from the polar effects of transposon insertions, that the transcriptional polarity must be from left to right and thus support our previous results (Braun et al. 1983; Gross et al. 1984). When the *Hin*dIII/*Sal*I fragment at the left-hand side of pRG12 was cut out (pRG108, not shown), no aerobactin precursor was synthesized which strengthens the conclusion that the promoter is in this region. When the remaining *Sal*I/*Eco*RI fragment was cloned into the pUC9 vector (pRG144), N-acetyl-N-hydroxy-lysine was made (Fig. 2), apparently as a result of use of the *lac* promoter. Transcriptional regulation of the *aer* region was studied quantitatively by Mud1(Ap *lac*) operon fusions (Braun and Burkhardt 1982). This has now been measured from the levels of mRNA produced from pColV-K30 with essentially the same results (Bindereif and Neilands 1985a). This procedure in addition demonstrated a major and minor transcriptional start site, at 30 and at about 80 base pairs, respectively, upstream of the initiation codon for the first structural gene which, according to our results, must be the *aerD* gene.

The close similarity of the aerobactin region on the two ColV plasmids K30 and K311 has recently been extended to other plasmids and to the chromosome of *E. coli* clinical isolates (Valvano and Crosa 1984; Bindereif and Neilands 1985b). The cloned aerobactin genes of pColV-K30 hybridized with certain plasmids and with chromosomal DNA fragments obtained by digestion with restriction enzymes. In addition, homologous aerobactin regions were identified in *Salmonella* and in *Shigella* species. Interestingly, inverted IS*1*-like elements were found to flank the aerobactin operons of *E. coli* (Perez-Casal and Crosa 1984; Bindereif and Neilands 1985b), *Shigella* (Lawlor and Payne 1984) and of *Salmonella* (McDougall and Neilands 1984; Colonna et al. 1985). The discovery of two replication regions at both ends of the ColV-K30 aerobactin region (Perez-Casal and Crosa 1984) suggests a mechanism for the high mobility of this region which could recombine via the IS*1*-like elements with IS*1* elements in plasmids and chromosomes. The rather frequent occurrence of the aerobactin region on invasive strains emphasizes the importance of this iron supply system for the virulence of the Enterobacteriaceae.

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